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Structure and morphology of vesicular dispersions based on novel glycophospholipids with various monosaccharide head groups

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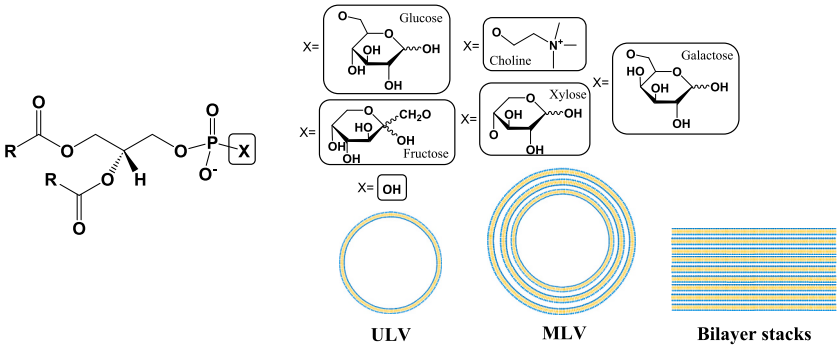
GRAPHICAL ABSTRACT



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ABSTRACT

Glycophospholipids combine the structural versatility of phospholipids and carbohydrates, but their potential as excipients and performance in other related applications remains largely unexplored due to their low natural abundance. We have synthesized four novel phosphatidyl saccharide conjugates with different carbohydrate head groups; glucose, galactose, fructose and xylose by using a Phospholipase D catalysed trans-phosphatidylation reaction. The combination of Small Angle X-ray Scattering (SAXS) and Cryogenic Transmission Electron Microscopy (cryo-TEM) data allowed us to characterize the dispersed glycophospholipid vesicles in excess water and under physiologically relevant solution conditions in terms of their morphology and structure. The different carbohydrate head group generated a large variability of the vesicle structures. Lipids conjugated with glucose and fructose self-assembled into unilamellar vesicles whereas galactose and xylose conjugated lipids formed multilamellar structures. Phosphatidylgalactose conjugated lipids formed a high

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number of stacked bilayers, while the phosphatidylxylose equivalent assembled into aggregates with only a few bilayers. These results highlight how carbohydrate hydroxyl spatial arrangements strongly influence lipid packing and self-assembly. The versatility of this glycophospholipid platform offers opportunities to generate biocompatible and biodegradable phospholipid excipients with properties that can be tailored for specific applications.

1. Introduction

Phospholipids (PLs) are naturally occurring polar lipids that self-assemble into nano-structures. They are therefore commonly used as formulation excipients in the cosmetic and pharmaceutical industries [1]. In excess water, the stacked bilayers or lamellar liquid crystalline phase can be dispersed to form vesicles. These are spherical structures consisting of one or several lipid bilayers curved around a water-filled cavity in the center. The vesicles can be loaded with cargo and the lipid bilayer(s) form a barrier that protects the cargo from enzymes, bile salts, free radicals and other degrading substances that are encountered upon administration to the human body [2,3].

However, when used for drug formulations, vesicular dispersions based on the most common PLs, such as phosphatidylcholine (PC), suffer from limited encapsulation capability of cargo, rapid clearance from the blood and poor storage stability. The physicochemical properties of the vesicles, such as size, number of bilayers, charge density, membrane fluidity, steric stabilization and permeability, are all factors that affect the properties of vesicles and how efficient they are as excipients for clinical applications [4]. Such properties are commonly altered by addition of new ingredients to the lipid mixture before the vesicles are prepared [5]. For example, cholesterol is added to reduce membrane fluidity and achieve better retention of cargo. Steric protectants, such as polyethylene glycol (PEG), can be used to increase vesicle colloidal stability and reduce the recognition by the mononuclear phagocyte system. Incorporation of a small portion of anionic lipid to the vesicle is another way to improve the stability due to electrostatic repulsion between the vesicles.

Apart from addition of these traditional ingredients to the formulation, the vesicles can be prepared with lipids that intrinsically possess the desired properties. One such candidate is phosphatidylinositol (PI), which is a naturally occurring phospholipid that is involved in transmembrane signalling and membrane fusion. It is a negatively charged phosphatidyl conjugate containing the highly hydroxylated, cyclic polyalcohol inositol in the polar part. Incorporation of PI in model membranes has been shown to dramatically alter the vesicle properties, such as membrane fluidity [6], metal cation induced fusion [7,8], and circulation time [9] compared to PC vesicles.

Another interesting type of lipid with similar properties to PI are glycophospholipids. The distinguishing feature of glycophospholipids is a carbohydrate moiety conjugated to the phosphate group in the hydrophilic part of the lipid. Similarly to PI, glycophospholipids possess a net negative charge and have a bulky, highly hydrated polar part, which is expected to increase the colloid stability of the vesicles through charge repulsion and steric stabilization. Glycophospholipids are a relatively unexplored type of lipid, which are naturally present in the form of phosphatidylglycosides, and only found in trace amounts, thereby limiting the possibility to study their phase behaviour and explore potential applications [10,11]. To produce high enough amounts of glycophospholipid for proper characterization, a synthetic approach has to be applied. This can be done in an efficient and selective way via an enzyme catalyzed reaction with Phospholipase D (PLD) [12–14]. However, the orientation of the saccharide of the resulting phosphatidyl saccharide conjugate will differ from the naturally occurring phosphatidylglycoside as it will be linked to the phosphate via a phosphodiester bond instead of a glycosidic linkage. Song et al. [15] were the first to report the physico-chemical properties of phosphatidyl saccharide vesicles. They showed that these lipids self-assembled into vesicles with increased long term colloidal stability and lower leakage of cargo compared to neat PC vesicles. This indicated that modifications with bulky, and highly hydroxylated compounds such as inositol and carbohydrates have a large impact on the lipid self-assembly.

In our previous study, we, for the first time, isolated high enough quantities of phosphatidyl-glucose lipids (P-Glu), to characterize pure P-Glu and PC/P-Glu mixtures in terms of Small Angle X-ray Scattering (SAXS) and Cryogenic Transmission Electron Microscopy (Cryo-TEM) measurements [16]. This study focused on changing the acyl-chain properties, from unsaturated oleic acid with fluid chains to saturated stearic acid derivatives. We found that P-Glu formed unilamellar vesicles (ULVs) in contrast to PC that primarily formed multilamellar vesicles (MLVs) using sonication. We also observed a transition towards less multilayer structures upon incorporation of P-Glu into PC vesicles. We believe that this transition is partly explained by the introduction of a negatively charged head group that induces electrostatic repulsion, but also by an increased flexibility of the bilayer due to the bulkiness of the head group. This is expected to increase undulation forces that in turn prevent formation of multi-layered structures. We did not observe any major effects of either temperature or variation of ionic strength, which indicate robustness of the vesicles and high potential as future excipient candidates.

In the present study, we have shifted the scope to focus on the effect of different saccharides in the head groups of the glycophospholipid, based on oleic acid acyl chains. Similarly to the previous study, we used SAXS and Cryo-TEM, to characterize the phospholipid conjugates which included a pentose (xylose) and hexoses (glucose, galactose, fructose), of which glucose, galactose and xylose are aldoses and fructose is a ketose. We also include a comparison with unmodified dioleoylphosphatidylcholine (DOPC) and the corresponding simplest anionic phospholipid: dioleoylphosphatidic acid (DOPA).

In water, carbohydrates enter a tautomeric equilibrium of open and closed ring forms. Upon ring closing, the stereochemistry of the anomeric carbon can take either the alpha- or beta configuration. Moreover, the closed structure can constitute either a five-membered ring (furanose) or a six-membered ring (pyranose). The tautomeric equilibrium differs among carbohydrates and is dependent on the properties of the surrounding medium. Fig. 1 illustrates the tautomeric equilibration of free monosaccharides in water at 25 °C for the four carbohydrates used for lipid conjugation. Since the different sugars differ in number and orientation of their hydroxyl groups and their tautomeric equilibrium of conformers, we have reason to believe that they will arrange in different ways, which will have pronounced effects on the bilayer structures.

2. Material and methods

2.1. Materials

1,2-Dioleoyl-sn-Glycero-3-Phosphatidylcholine (DOPC) > 98 % purity and 1,2-Dioleoyl-sn-Glycero-3-Phosphatidic acid (DOPA) > 98 % purity were purchased from Larodan (Solna, Sweden). For the lipid synthesis PLD from *Streptomyces* sp. was purchased from Sigma Aldrich, and glucose, galactose, fructose and xylose of >99 % purity were purchased from Sigma Aldrich. Chloroform ($CHCl_3$), methanol (MeOH) (99 %) and glacial acetic acid were purchased from VWR Chemicals. The PBS buffer was prepared from NaCl, KH_2PO_4 and Na_2HPO_4 (99.9 %), which were purchased from VWR Chemicals. Milli Q purified water (18

MΩ cm) was used for all experiments.

3. Methods

3.1. Synthesis of modified phospholipids

Glycophospholipids were synthesized by a PLD-catalyzed trans-phosphatidylation reaction as described by Barchan et al. [14]. In short, the synthesis was performed in a two-phase reaction system consisting of chloroform, containing the donor PL, and an aqueous solution, acetate-phosphate buffer pH 5.6, containing the acceptor substrate (glucose, galactose, fructose or xylose) and PLD enzyme. The reactions were run for 24 h with 50 times excess of acceptor substrate. After the reaction was complete, the modified lipids were isolated through a combination of chloroform extraction and flash chromatography. Excess sugar was removed by a simple extraction procedure with chloroform and lipids accumulated in the organic phase. After centrifugation, the organic phase was recovered, and the solvent was removed by evaporation. The lipids were then re-dissolved in $CHCl_3$:MeOH (93:7, *v*/v) and purified by flash chromatography on a silica column. After washing with 3 column volumes (CV) of chloroform, the lipids were eluted with a linear gradient of 10 CV spanning from 100 % chloroform to 100 % $CHCl_3$:MeOH:HAc (8:3:1, v/v). Fractions containing the purified lipid were pooled and concentrated by evaporation before lyophilization.

3.2. Preparation of vesicles

In this study, the vesicle morphology and characteristics of the four modified glycophospholipids dioleoylphosphatidyl-glucose (DOP-Glu), −galactose (DOP-Gal), −fructose (DOP-Fru) and -xylose (DOP-Xyl), as well as two reference lipids, DOPC, which is the unmodified lipid used as starting material for the synthesis, and DOPA, which is the simplest anionic phospholipid, were invesitgated. Vesicles were prepared with the adapted method based on the protocol described by Dabkowska et al. [17]. Stock solutions of each lipid were prepared in 2:1 (*v*/v) ratio of $CHCl_3$:MeOH from which lipid films with a nominal mass of 10 mg were made by gentle evaporation of the solvent using N_2 gas. The lipid films were then hydrated to a final nominal concentration of 10 mg/mL in MilliQ water and vortexed until fully suspended. The final estimated lipid concentrations for each sample are displayed in Table 1. The lipid suspensions were sonicated in a water bath using a tip sonicator (Vibra-Cell VCX 130, Sonics & Materials Inc., Newton, CT, USA) with the following settings: 7.5 min total sonication time, divided in pulses of 10 s sonication and 10 s cooling time at 50 % amplitude with 3 mm stepped sonication tip.

3.2.1. Effect of salt

The effect of increased ionic strength on vesicle morphology was investigated by addition of 20 times concentrated PBS (20× PBS) to the vesicles dispersed in water. The final ion concentration in the lipid samples was 155 mM NaCl, 1.06 mM KH_2PO_4, 2.97 mM Na_2HPO_4, pH 7.3. The diluting effect (of 5 % *v*/v) on the lipid concentration induced by addition of 20× PBS was considered negligible.

Table 1
Final lipid concentrations in stock vesicle samples.

Lipid	Concentration in SAXS experiments
DOPC	10 mg/mL
DOPA	10 mg/mL
DOP-Glu	10 mg/mL
DOP-Gal	10 mg/mL
DOP-Fru	<20 mg/mL*
DOP-Xyl	10 mg/mL

* Estimated lipid concentration as a completely dry lipid sample was not possible to obtain.

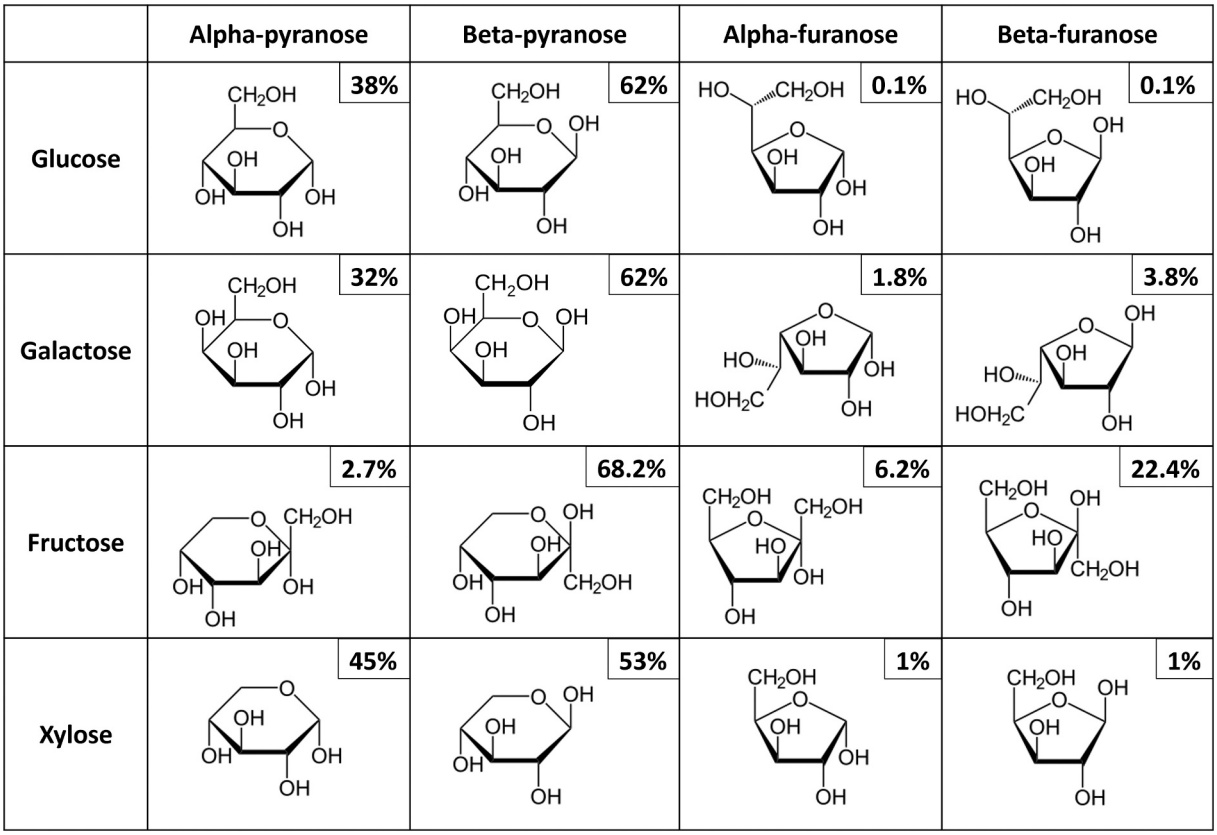

Fig. 1. Chemical structures of monosaccharide tautomers in water. The number in the upper right corner displays the abundance in water at 25 °C.

3.3. Synchrotron SAXS

SAXS measurements were performed at the CoSAXS beamline, MAX IV (Lund, Sweden) using the sample autoloader into a quartz capillary (diameter = 1.5 mm) and an Eiger2 4M SAXS detector at a distance of 5 m from the sample. Measurements were performed at 25 °C and 37 °C, controlled by setting the sample temperature in the loading plate to 25 °C (actual = 25.5 ± 0.1 °C) and 37 °C (actual = 36.8 ± 0.3 °C) for the temperature scan. For each sample, 400 frames were collected with an exposure time of 20 ms per frame over a q range of 0.0024–0.36 $Å^{-1}$. The 2D SAXS data were reduced, frame averaged, normalised and background subtracted by using the MatFRAIA algorithm provided by MAX IV [18].

3.4. Data modelling

SAXS data were fitted using the procedure described by Pal et al. [19]. The procedure for data modelling is described in detail with the relevant equations by Barchan et al. [16] based on the method described by Pabst et al. [20]. Briefly, the form factor can be obtained as the square of the Fourier transform electron density profile (EDP), that describes the bilayer. Here we combine two Gaussians of width σ_H, centered at $z = \pm z_H$, to represent the head groups, and a third Gaussian to represent the terminal methyl groups at the bilayer center ($z = 0$). The Gaussian representing the head group of the inner and outer leaflets are assumed to be similar and the electron densities are scaled based on the methylene bilayer mid plane electron density. The structure factor of the multilamellar systems with repeat distance, d, was obtained by using a modified Caillé model. We define the bilayer thickness as $d_B = 2(z_H + \sigma_H)$ with water layer thickness $d_w = (d - d_B)$ and the values were all obtained from the model fitting parameters.

3.5. Cryo-TEM

Vesicle morphology was studied with a JEM-2200FS transmission electron microscope, located nCHREM at Lund University, operated at 200 kV. The images were recorded with a TemCam-F416 camera (TVIPS) using SerialEM software. The sample (4 μL) with the lipid dispersion was blotted for three seconds on the non-coated side to remove excess liquid before transferred to plasma cleaned and hydrophilized (GloCube Quorum) Cu grids coated with a lacey-carbon film (Ted Pella). The grids were then plunged into liquid ethane (−184 °C) using an automatic plunge freeze system (Leica EM GP) for rapid vitrification. The plunged grids were stored in liquid nitrogen (−196 °C) until transferred by a cryotransfer tomography holder (Fischione Model 2550) for imaging under the microscope.

4. Results and discussion

A recently developed synthesis method made it possible to prepare a set of phospholipids with various saccharides as the polar groups. The method takes advantage of the broad specificity of *Streptomyces* sp. Phospholipase D with respect to potential nucleophiles in the transphosphatidylation of phosphatidyl choline. Four different monosaccharides (glucose, galactose, fructose and xylose) were conjugated to the phospholipid head group to produce dioleoylphosphatidyl glucose (DOP-Glu), dioleoylphosphatidyl galactose (DOP-Gal), dioleoylphosphatidyl xylose (DOP-Xyl) and dioleoylphosphatidyl fructose (DOP-Fru). Conjugation of the phosphatidyl group to a carbohydrate instead of choline changes the charge from the previously zwitterionic DOPC into an anionic phosphatidyl saccharide lipid. The synthesized lipids were dispersed in Milli Q water via tip sonication to form vesicles, which were characterized by dynamic light scattering (DLS), presented in Table S1. The average hydrodynamic diameters varied between 80 and 400 nm. These vesicle structures were compared to the structures formed by the unmodified lipid (DOPC) and the simplest anionic phospholipid (DOPA). Subsequent analysis with SAXS and cryo-TEM confirmed the formation of a variety of different structures. A comparison between vesicle preparation via tip sonication and extrusion for DOP-Glu is included in Fig. S4. A stability test indicated the DOP-Glu vesicles remained virtually unchanged after storage for 5 days at 4 °C (Table S2).

To discuss the structures formed, it can be useful to consider the concept of the critical packing parameter (CPP) introduced by Israelachvili et al. (1976) [21].

$$\mathrm{CPP} = \frac{v}{a_0 l_c}$$

where v is the volume of the hydrocarbon tail, a_0 is the optimal surface area per amphiphile and l_c is the (critical) length of the hydrocarbon chain. Here, micelles would have a CPP < 1, lamellar structures CPP ≈ 1 and inverse structures, like inverse cubic, hexagonal and micellar, CPP > 1. In our study, v is expected to be constant and to a first approximation we only vary a_0. As we mainly observed bilayer type structures we should expect CPP ≈ 1. The concept of packing parameter was further developed for vesicles by Mitchell and Ninham (1981) [22]. They show the effect of the packing constrains as the vesicle diameter decreases. In their approach the number of lipid molecules in the inner leaflet decreases relative to the number in the outer leaflet as the size of the vesicles decreases. The apparent value of a_0 increases and the CPP decreases. For a flat bilayer the packing parameter is 1 and a_0 is at its minimum. Judging from the DLS data in Table S1, the headgroup area should increase in the order DOP-Gal < DOP-Xyl< DOPC < DOP-Glu ≈ DOP-Fru < DOPA. The a_0 and l_c are strictly not independent but coupled and will be reflected in the bilayer thickness as will be discussed further below.

4.1. Vesicle morphologies

From the obtained SAXS profiles of the vesicles, it was possible to determine the vesicle morphology and lamellarity. Fitting of the form factor model to the SAXS data and extraction of the electron density profile gave a deeper insight into the vesicle structure and the bilayer parameters. The observed results were further confirmed by cryo-TEM imaging.

The zwitterionic reference lipid DOPC formed MLVs with a high number of correlated bilayers as indicated in Fig. 2A (data replotted from [16]). On the contrary, anionic DOPA formed smaller ULVs as apparent from both SAXS data and cryo-TEM imaging, Fig. 2B. We also note that the DOPA ULVs were mixed with some long fibrous structures as shown in Fig. S1A.

The conjugation with different saccharide head groups resulted in distinctively different vesicle structures in terms of shape and lamellarity depending on the saccharide head group, as displayed in Fig. 3. DOP-Glu (Fig. 3A, replotted from [16]) and DOP-Fru (Fig. 3C) both formed small uniform ULVs with similar scattering patterns and electron density profiles (EDPs). The presence of a homogenous population of ULVs was indicated by the broad uniform peak centred around q = 0.1 $Å^{-1}$ in the SAXS data. In contrast, the DOP-Gal scattering pattern features a sharp and narrow peak at q = 0.1 $Å^{-1}$ that indicates MLVs and/or bilayer stacks with a high number of correlated bilayers (Fig. 3B). Similarly, DOP-Xyl (Fig. 3D) also displayed a sharp peak at q = 0.1 $Å^{-1}$. However, in this case the scattering pattern, in addition to the sharp peak, features the broad peak of the bilayer form factor observed for DOP-Glu and DOP-Fru. This suggests MLVs as the dominating structure, but with a lower number of correlated bilayers.

The cryo-TEM images (Fig. 3) are consistent with the SAXS data regarding the observed vesicle morphologies. Both DOP-Glu and DOP-Fru displayed fairly monodisperse small, uniform ULVs (Table S1). The cryo-TEM images of the DOP-Xyl dispersions featured MLVs, although with a lower number of correlated bilayers compared to DOP-

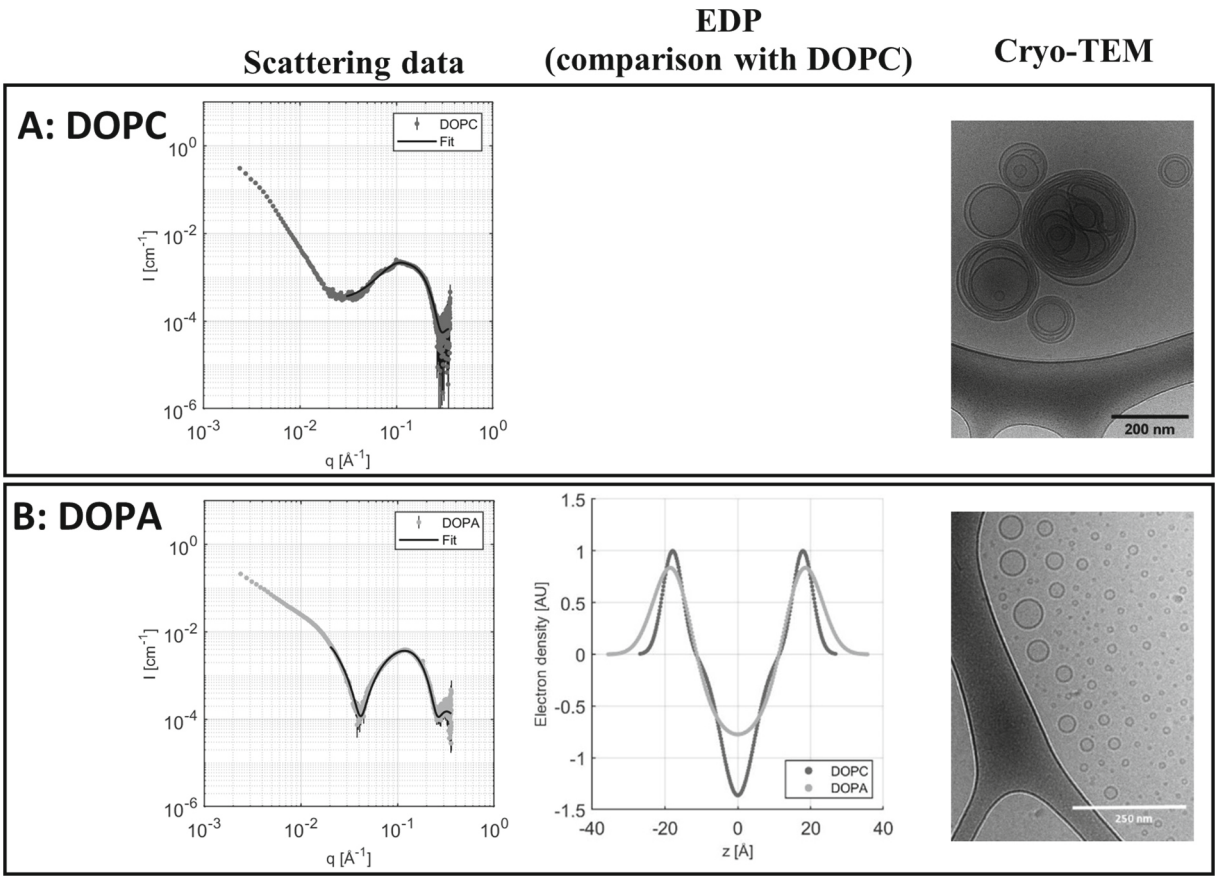

Fig. 2. SAXS data (left), Overlaid DOPC (black line) and DOPA (gray line) electron density profiles (middle) and cryo-TEM images (right) of vesicles of reference lipids. A: DOPC, B: DOPA. Data for DOPC are replotted from Barchan et.al [16] (obtained under identical conditions). The black lines in the SAXS plots show the fit of the scattering profile with the models described by e.g. Pal et al. [19].

Gal, which agrees well with the morphological differences indicated by the SAXS data. DOP-Gal dispersions showed MLVs with several correlated bilayers and almost no presence of ULVs. The cryo-TEM images show that in addition to typical ULVs and MLVs more irregular structures were formed as well. It should be kept in mind that multilamellar structures, like bilayer stacks, were less efficiently transferred to the grid for cryo-TEM studies, therefore, the cryo-TEM results were not used for quantitative assessment of various types of morphologies. However, using the SAXS data it was possible to estimate the average number of correlated bilayers, as described in the section "Bilayer parameters" below. It has been reported that small angle neutron scattering (SANS) can be used to obtain detailed information on the proportions of uni- bi and multilamellar species in preparations of PC based vesicles [23].

From the observed vesicle morphologies, it is clear that the conjugation of different carbohydrates to the phospholipid head group resulted in dispersions of distinctly different structures and shapes, which is schematically illustrated in Fig. 4. This implies that the number and spatial orientation of hydroxyl groups in the polar head play a critical role in lipid self-assembly structures. As the carbohydrate part of these lipids enters a tautomeric equilibrium when dissolved, the surrounding medium conditions dictate the final lipid molecular structure. This means that chemically pure glycophospholipid materials will behave like lipid mixtures with an equilibrium composition that could significantly vary depending on the carbohydrate head groups. As a result, different isomers and mixtures thereof can lead to distinct lipid assembly properties, despite their overall similar chemical structures. A recent detailed investigation of the chemical structure of the phosphatidyl saccharide conjugates used in the current work, using a combination of mass spectrometry (MS) and nuclear magnetic resonance (NMR), confirms the existence of different isomers [24]. The importance of small changes in the molecular structure has been demonstrated for the self-assembly structure of alkylglycoside $C_{16}G_2$, which has been studied with scattering techniques and cryo-TEM [25]. Here the anomeric configuration strongly affected the micellar structure formed, as β-$C_{16}G_2$ was found to form elongated wormlike micelles, whereas α-$C_{16}G_2$ formed shorter cylindrical micelles. The results were interpreted as an effect of sugar head group interactions, where the β-$C_{16}G_2$ anomer could pack more efficiently than the α-$C_{16}G_2$.

It has been reported previously in a study of synthetic branched chain glycosides, that the glucose derivative formed reversed hexagonal structures, while the maltose derivative due to its larger polar group formed lamellar vesicles. These vesicles were multilamellar, but a shift to ULVs could be obtained by addition of dodecyl sulfate or Aerosol OT [26]. Modification with PEG has been widely used to increase the circulation time of phospholipid-based vesicles when used for drug delivery. The PEG reduces the binding of serum components and also causes steric repulsion, preventing the aggregation of the vesicles [27]. Furthermore, it has been observed that as little as 0.1 mol% PEG-ylated lipid caused a shift from MLVs to ULVs for phospholipid-based vesicles [23]. In the present study we demonstrate that either ULVs or multilamellar structures with various numbers of correlated bilayers can be obtained by proper choice of monosaccharide group in glycophospholipids having oleic acid residues as the nonpolar part. In our previous study, we showed that an exchange of oleic acid in DOP-Glu with the saturated palmitic or stearic acid caused an increased tendency to form multilamellar structures, and we speculate that a similar modification of the other glycophospholipids in the present study would also lead to increased formation of MLVs and stacks. Tailoring a glycophospholipid with respect to saccharide head group and fatty acid can thus be used to give access to a very wide range of vesicular structures.

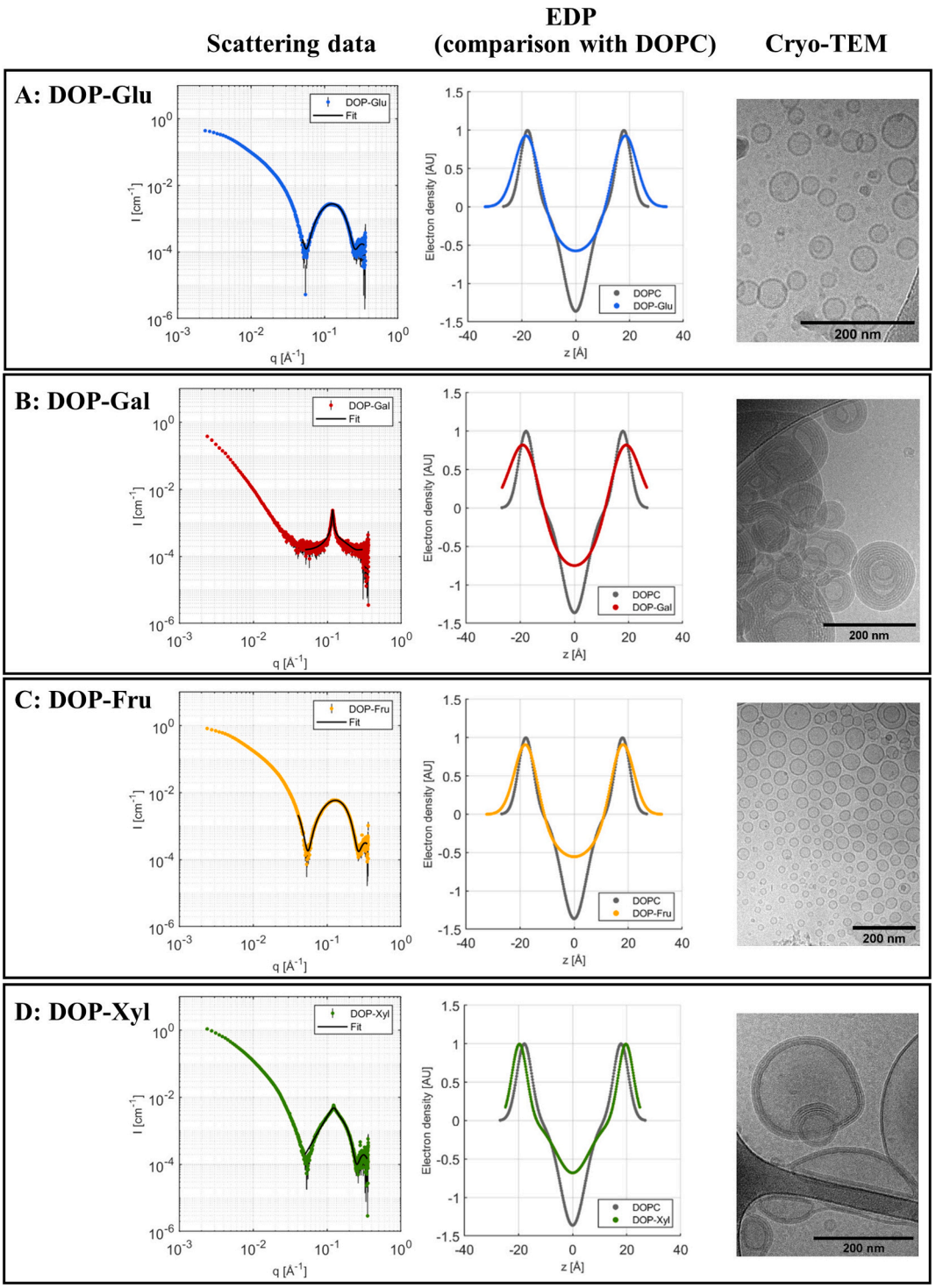

Fig. 3. SAXS data (left), electron density profiles (middle) and cryo-TEM images (right) of vesicles of carbohydrate modified lipids. A: DOP-Glucose, B: DOP-Galactose, C: DOP-Fructose, D: DOP-Xylose. Data for DOPC and DOP-Glu are replotted with permission from Barchan et.al [16] (obtained under identical conditions). The black lines in the SAXS plots show the fit of the scattering profile with the models described by e.g. Pal et al. [19]. DOPC (gray line) in EDP plots is added for comparison.

4.2. Bilayer parameters

The fact that lipids modified with carbohydrates in the head group displayed dramatically different vesicle morphologies suggests that also the bilayer thickness could be affected by the type of head group due to the different packing of the lipid molecules in the bilayer.

The thickness and other bilayer parameters can be extracted based on model fitting to the SAXS scattering profile as described by e.g. Pal et al. [19] and Pabst et al. [20]. The bilayer thickness in terms of $2z_H$ (see Materials and Methods section) can be directly extracted from the electron density profile. Due to the high scattering contrast, i.e. difference in electron density between the phosphate group and surrounding medium, it gives a reliable and consistent estimation of bilayer thickness. The reference lipids and all carbohydrate modified lipids except for DOP-Xyl had a similar head-to-head distance of around 36 Å, while DOP-Xyl had a somewhat larger distance of 39.4 Å, as shown in Table 2. This

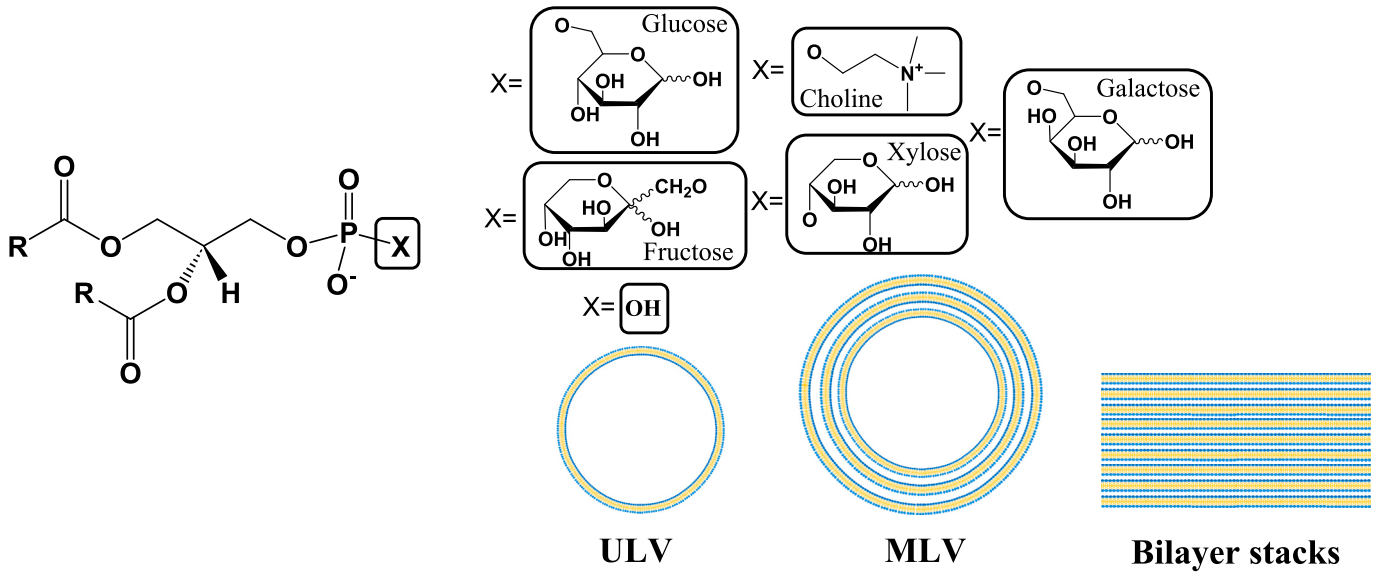

Fig. 4. The self-assembled lipid bilayer structure is influenced by the chemical structure of the phospholipid head group.

Table 2
Properties of vesicles of DOPC, DOPA and modified DOP-saccharide lipids based on the SAXS and Cryo-TEM data. Data for DOPC and DOP-Glu are reproduced from Barchan et.al [16] (obtained under identical conditions).

Lipid	Dominant morphology	$2z_H$ [Å]	d_B [Å]	d [Å]
Unmodified lipids				
DOPC	MLV	35.8 ± 1.3	41.3 ± 2.9	53.7 ± 0.5
DOPA	ULV	35.8 ± 0.6	45.9 ± 1.6	–
Modified lipids				
DOP-Glu	ULV	36.2 ± 2.6	45.1 ± 2.8	–
DOP-Gal	High N MLV/stack	36.6 ± 0.4	47.6 ± 1.0	53.4 ± 0.1
DOP-Fru	ULV	35.5 ± 0.6	43.9 ± 1.4	–
DOP-Xyl	Low N MLV	39.4 ± 1.0	44.9 ± 2.4	49.7 ± 0.1

indicated that the type of head group modification of the lipid did not induce any major changes to bilayer thickness despite the large variation in vesicle morphology across the samples. The value obtained for DOPC agrees with earlier reports [28,29].

The higher value for DOP-Xyl indicates a thicker bilayer, which appears to be contradicted by the fact that based on the molecular structure (Fig. 1) this should be the smallest sugar group. However, NMR data suggest that conjugation of xylose to the phosphate occurs via secondary hydroxyl groups, in contrast to the other lipids which are conjugated via primary hydroxyls [24]. This implies that the head group interaction and hence area per molecule could be different and lead to a thicker bilayer.

In our previous study [16], we observed that the bilayer thickness increased with the effective acyl chain length for both PC and the corresponding PL modified with glucose in the head group. The introduction of glucose significantly changed the self-assembled structure and the resulting bilayer parameters, where glucose gave ULVs rather than MLVs and a larger bilayer thickness compared to choline analogues. The carbohydrate moieties are larger than a choline group and the presence of several hydroxyl groups is expected to increase hydration. Both these factors are expected to lead to a thicker lipid bilayer.

It is noteworthy that the saturated lipids, dipalmitylphosphatidyl-glucose (DPP-Glu) and distearylphosphatidyl-glucose (DSP-Glu), were more affected by the new head group and resulted in a larger increase in bilayer thickness compared to mono-unsaturated DOP-Glu. The saturated lipids were studied below their phase transition temperature and were thus in the lipid gel phase, in contrast to DOP-Glu which was in the fluid crystalline phase. This observation indicates that the bilayer thickness was mainly controlled by the acyl chains, especially for lipids in fluid state, but that the head group plays a contributing role for bilayer thickness of vesicles where the lipids are in gel phase. Lipids in the gel phase have a stronger membrane rigidity, which could affect the bilayer thickness.

The width of the head group region, which can be deduced from the overall bilayer thickness, d_B, where $d_B = 2(z_h + \sigma_h)$ with σ_h being the width of the Gaussian describing the head group EDP [16], is expected to be more sensitive to the head group variation than the overall bilayer thickness. The zwitterionic DOPC had the smallest d_B of 41.3 Å, which can be explained by its smaller size and lower hydration of the choline head group compared to the carbohydrates. The negatively charged lipids had a larger d_B ranging from 43.9 to 47.1 Å in the order of DOP-Fru < DOP-Xyl < DOP-Glu < DOPA < DOP-Gal, Table 2. However, it is important to consider that this value is more uncertain than the one deduced from maxima of the EDP.

Among the lipids forming MLVs, the lamellar distance, d (i.e. the bilayer plus the thickness of the water layer between two bilayers), were similar for DOPC and DOP-Gal, and slightly smaller for DOP-Xyl. The average number of correlated bilayers were on the order of 15 for DOP-Gal and 3–4 for DOP-Xyl as indicated in Table 2. Cryo-TEM imaging for DOP-Xyl agrees well with these findings. Cryo-TEM images for DOP-Gal showed MLVs with approximately 8–10 correlated bilayers, which is lower than calculated from the SAXS fit. It is probable, however, that each population of lipid particles in the DOP-Gal sample were not equally transferred to the grid, due to the sample preparation procedure for cryo-TEM. Particles with a considerably higher number of correlated bilayers are generally larger and either separate out before transfer to the grid or are not so efficiently transferred. Moreover, long fibrous structures alongside the vesicles were detected in the cryo-TEM imaging, which could potentially be bilayer ribbons, but it remains unclear if these structures are contaminations or an artefact of cryo-TEM sample preparation (Fig. S1B). Somewhat similar structures, identified as threadlike micelles, have been detected in dispersions of egg

phosphatidyl choline with high content of lyso-phosphatidyl choline, as previously reported by Bergstrand et al. [30]. Interestingly, both the type and size of structures formed in such systems have been shown to depend on the preparation path [31,32]. Thus, it seems that aggregates that are kinetically trapped can appear, and the samples do not represent equilibrium structures for MLVs with large numbers of bilayers.

4.3. Effect of ionic strength on vesicle structure

The addition of salt to lipid dispersions can have large effects on the self-assembly of charged lipids as it affects the electrostatic interaction between the vesicles or within the vesicular structure due to screening of these interactions when increasing the ionic strength. The salt solution was added after the formation of the vesicles and the transportation of electrolytes across the bilayers is expected to be limited, thus there will be a concentration gradient of ions across the lipid bilayer invoking an osmotic stress as discussed by Piccinini et al. based on small angle neutron scattering data of PC vesicles [33]. For the reference lipid DOPA, the addition of PBS induced a clear change in the scattering data with the emergence of a second peak on the low q side, resembling a scattering pattern from sponge phase nanoparticles [34] (Fig. S2b). This could be the result of charge screening that reduces the effective head group cross section as well as inducing an osmotic stress that results in a shift from ULVs towards structures with more negative curvature. A vesicle-to-sponge transition has previously been reported being due to increased proportion of eicosapentaenoic acid (EPA) in monoolein/EPA mixtures [35].

The salt effect on DOPC vesicles, as reported by Barchan et al. [16], induced slightly more ordered bilayers as indicated by a small increase in peak definition of the correlation peak. It also presented a small shift towards higher q, indicating a decreased interbilayer distance (Fig. S2a).

Regarding the modified lipids, the salt effect was less significant. For multilamellar DOP-Gal (Fig. S3b) and DOP-Xyl (Fig. S3d) vesicles, no effect was observed. DOP-Fru (Fig. S3c) showed a small shoulder on the low q side of the form factor peak, alternatively the emergence of a second peak, similarly to the peak observed for DOPA vesicles. In comparison, DOP-Glu vesicles, reported by Barchan et al. [16], were not affected by PBS (Fig. S3a).

Finally, we note that no effect of elevated temperature (37 °C) was observed for any of the lipid dispersions.

5. Conclusions

5.1. Key findings

We show that conjugation of different carbohydrates to the lipid head group can be used to control the lipid self-assembly structures, providing ULVs and MLVs of varying sizes and degrees of lamellarity. Here, dioleoylphosphatidyl-glucose (DOP-Glu) and -fructose (DOP-Fru) formed ULVs, as verified by both cryo-TEM and scattering data. Dioleoylphosphatidyl-galactose (DOP-Gal) and -xylose (DOP-Xyl) formed multilamellar structures, in which DOP-Gal had a high number of correlated bilayers whereas DOP-Xyl vesicles only had a few bilayers. Interestingly, DOP-Glu and DOP-Gal, the most similar lipids with regards to chemical structure, showed the largest difference in vesicle morphology. A contributing reason for this could be the complex chemistry of carbohydrates in solution, that results in a tautomeric equilibrium of alpha/beta and furanose/pyranose configurations. The surrounding medium dictates the composition of this equilibrium, which could be considerably different for different carbohydrates. In the case of fructose, one should also take into consideration that the link to the lipid can occur via either the primary hydroxyl group on carbon-1 of the monosaccharide or the one on carbon-6 [24].

5.2. A new concept for lipid modification

The combination of structurally diverse phospholipids and carbohydrates gives rise to glycophospholipid conjugates with substantial variability. However, their limited natural abundance has left their potential as formulation excipients and use in other functional applications largely unexplored. By providing a new procedure to synthesize a wide range of structurally different carbohydrate head groups we have laid the foundation for a diverse platform of glycophospholipids with distinctly different properties. This was achieved by phospholipase D catalyzed transphosphatidylation reactions, which provide the ability to adjust head group composition. In turn this allows for precise tuning of membrane dynamics, and provide potential for glycophospholipids as valuable excipients in applications such as drug delivery, bioengineering and functional food formulations.

5.3. Key advances compared to previous studies

Our previous study focused on the effect of the acyl-chain properties, fluidity and chain melting point [16]. This study explores the impact of different saccharide head groups on glycophospholipids constructed with oleic acid acyl chains as a fixed hydrophobic component. We have used a phospholipase D catalysed transphosphatidylation reaction to synthesize four novel phosphatidyl saccharide conjugates with different carbohydrate head groups; glucose, galactose, fructose and xylose. Song et al. [15], were among the first to report on the physico-chemical properties of phosphatidyl saccharide vesicles and showed that they increased long term colloidal stability and provided lower leakage of cargo compared to neat phosphatidylcholine vesicles. Here we extended the structural characterization by combining SAXS and cryo-TEM. The obtained data allowed us to characterize the dispersed glycophospholipid vesicles in terms of their morphology and structure in water and under physiologically relevant solution conditions.

5.4. Vision for future work

This study highlights how carbohydrate hydroxyl spatial arrangements strongly influence lipid packing and self-assembly. Our vision is to develop the synthesis procedure so that larger quantities can be obtained to also provide insight into more concentrated systems. Here non-lamellar lipid nanoparticles are particularly important. We envision that the generated lipids can replace the PEG-ylated lipids as a more controllable and sustainably produced compound. The study suggests that the solution conditions are important and clearly calls for more thorough studies of different physiologically relevant conditions. Furthermore, MD simulations are highly motivated, to understand why such similar carbohydrate parts of glycophospholipids can cause the formation of such different vesicular structures. In order to evaluate the potential of the new materials for drug delivery applications, it will be of crucial importance to study aspects such as encapsulation efficiency and kinetics of leakage/release, as well as interactions with biomolecules and biocompatibility.

CRediT authorship contribution statement

Nikolina Barchan: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jennifer Gilbert:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Antara Pal:** Writing – review & editing, Formal analysis, Data curation. **Tommy Nylander:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Patrick Adlercreutz:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2025.139585.

Data availability

Data will be made available on request.

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